3063

Lipid Metabolism

EVIDENCE OF A δ-OXIDATION PATHWAY FOR SATURATED FATTY ACIDS

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1. Specific radioactivities of milk triglyceride fatty acids and γ- and δ-hydroxy fatty acids were measured after the intramammary infusion of [1-^{14}C]acetate, δ-hydroxy[1-^{14}C]laurate and [1-^{14}C]laurate as their sodium salts into fed lactating goats. 2. Net incorporations of the radioactive tracer into the total milk lipids were comparable, being 16, 17 and 21% of the label infused respectively. 3. The specific radioactivities of the C_4–C_8 fatty acids after [1-^{14}C]acetate infusion were lower than those of the C_{10}–C_{14} fatty acids. 4. After δ-hydroxy[1-^{14}C]laurate administration the milk triglyceride fatty acids were labelled and their specific radioactivities were characterized by decreasing values with increasing chain length of the fatty acids, implicating C_4 unit incorporation. 5. The γ- and δ-hydroxy fatty acids isolated after [1-^{14}C]laurate infusion were highly labelled and the milk triglyceride fatty acids, other than laurate, exhibited a labelling pattern similar to that of the fatty acids derived from the radioactive δ-hydroxy fatty acid. 6. Evidence is presented for the existence of saturated fatty acid δ-oxidation in the mammary gland, in which the γ- and δ-hydroxy fatty acids are active intermediates.

Numerous studies from our own and other Laboratories have provided evidence of aliphatic γ- and δ-lactones in mammalian lipid systems (Boldingh & Taylor, 1962; Dimick, Patton, Kinsella & Walker, 1966*b*). The immediate precursors of these trace compounds have been identified as the corresponding γ- and δ-hydroxy fatty acids, which are esterified to glycerol in the form of monohydroxyacyl triglycerides (Wyatt, Pereira & Day, 1967; Jurriens & Oele, 1965). Lactones are formed from the latter by hydrolysis of the unstable hydroxyacyl ester bond and subsequent loss of water from the liberated hydroxy fatty acids. Recent efforts in this Laboratory have been directed towards elaboration of the biosynthetic origin of γ- and δ-hydroxy fatty acids and the determination of their role in mammalian lipid metabolism. Walker, Patton & Dimick (1968) have demonstrated that [1-^{14}C]acetate is incorporated *in vivo* into the δ-hydroxy fatty acids of goat milk fat. Their results implied that these hydroxy acids are produced from acetate in a manner similar to biosynthesis of the corresponding saturated fatty acids. The present study was undertaken to further elucidate the biochemical pathway of γ- and δ-hydroxy fatty acid biosynthesis. Evidence is presented for the existence of saturated fatty acid δ-oxidation, which results in the formation of these hydroxy fatty acids.

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MATERIALS AND METHODS

^{14}C-labelled acids. Sodium [1-^{14}C]acetate was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. [1-^{14}C]Lauric acid was obtained from Tracerlab, Waltham, Mass., U.S.A. [1-^{14}C]δ-Dodecalactone was kindly supplied by Dr J. Boldingh, Unilever Research Laboratory, Vlaardingen, The Netherlands. Each of the labelled substrates was checked for radiochemical purity on a Barber–Colman model 5000 gas chromatograph equipped with a model 5190 radioactive monitoring system for detection of organic ^{14}C as $^{14}CO_2$. The [^{14}C]lauric acid and [^{14}C]lactone were converted into their sodium salts by refluxing gently in 0·5N-NaOH. Each labelled substrate was dissolved in 5ml. of water for infusion into the mammary gland via the teat canal.

Sampling procedure. Healthy lactating goats, each producing approx. 1500ml. of milk/day, were used in this study. After complete milking, the labelled substrate (Table 1) was administered by intramammary infusion. Milk samples were collected at specified time-intervals. Each goat secreted approx. 100ml. of milk/hr., indicating highly active secretory tissue. The lipid was recovered from the milk samples by the Roese–Gottlieb solvent extraction procedure (Association of Official Agricultural Chemists, 1960, p. 190).

Analysis of γ- and δ-hydroxy fatty acids. Silicic acid adsorption chromatography was employed to isolate the monohydroxyacyl triglycerides from the radioactive milk samples as previously reported (Walker *et al.* 1968). The γ- and δ-hydroxy fatty acids were liberated from the glycerides by saponification and acidification for qualitative and quantitative analyses as their corresponding lactones by gas–liquid chromatography. Quantitative studies (Jurriens & Oele, 1965; Dimick & Walker, 1967) utilizing these procedures provide evidence of 95–98% recovery of the hydroxy acids as lactones.

Analysis of fatty acids. Approx. 4g. of triglyceride isolated from each lipid sample by the above procedure was distilled according to the Reichert–Meissl technique (Association of Official Agricultural Chemists, 1960, p. 364). This allowed an increase in concentration of the water-soluble fatty acids for further analysis. Both the water-soluble and insoluble fatty acids were quantitatively measured as their methyl esters (Metcalfe, Schmitz & Pelka, 1966) by gas–liquid chromatography.

Assay of radioactivity. The individual methyl esters of the ^{14}C-labelled fatty acids (C_6–C_{18} acids) were isolated by trapping from the carrier gas effluent. Trapping was accomplished by attaching a 12in. × 0·25in. U-shaped glass tube, filled with washed sand, via a hypodermic needle to the heated (250°) outlet of the column. These traps were cooled to −70° in ethanol–solid CO_2 during the trapping procedure until elution with scintillation fluid via the syringe needle directly into counting vials for assay in a Packard Tri-Carb liquid-scintillation spectrometer.

Methyl butyrate, because of its volatility, was quantitatively isolated for radioactivity assay by the column-chromatographic procedure of Keeney (1956).

Specfic radioactivities (counts/min./mg.) of the radioactive fatty acid esters and radioactive γ- and δ-lactones were calculated by using the mass data (weight percentage distribution) and the radioactivity distribution. Gas–liquid radiochromatography was also utilized to confirm the ^{14}C radioactivity in the various compounds isolated during the study.

RESULTS

Sodium [1-^{14}C]*acetate infusion.* Results of this phase of the study have been partially reported by Walker *et al.* (1968). However, it is pertinent to discuss them in conjunction with the present study. The specific radioactivities of the saturated fatty acids (Fig. 1) are in accord with those of other workers who have studied, *in vivo*, the biosynthesis of fatty acids from acetate in the mammary gland of the ruminant (Popják, French, Hunter & Martin, 1951; Lawrence & Hawke, 1966). The specific radioactivities of the C_4, C_6 and C_8 fatty acids were substantially lower than those of the C_{10}, C_{12} and C_{14} saturated fatty acids. The lower specific radioactivity of palmitate is understandable in view of the significant contribution of blood plasma palmitate to the milk fatty acids in the gland (Dimick, McCarthy & Patton, 1966*a*; Annison, Linzell, Fazakerly & Nichols, 1967).

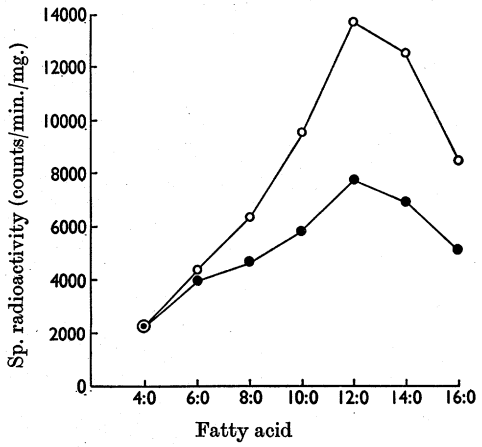


Fig. 1. Expt. 1: specific radioactivity of milk triglyceride fatty acids after intramammary infusion of sodium [1-^{14}C]-acetate. Samples were taken 5hr. (●) and 10hr. (○) after administration of [1-^{14}C]acetate.

The specific radioactivities of the δ-hydroxy acids (as δ-lactones) throughout the experiment after sodium [1-^{14}C]acetate administration indicated slightly lower values than their corresponding fatty acids (Walker *et al.* 1968). These results imply that the δ-hydroxy acids are products of the individual fatty acids and not intermediates in their formation.

Sodium δ-hydroxy[1-^{14}C]*laurate infusion.* Incorporation of the ^{14}C into the total milk fat collected after the administration of δ-hydroxy[1-^{14}C]laurate was comparable with the incorporation of [1-^{14}C]-acetate (Table 1). The specific radioactivity of the milk-fat triglyceride fractions increased from the 3hr. sample to a maximum in 6hr. and then decreased in the 9hr. sample. Analyses of the hydroxyacyl glyceride fractions from each milking indicated that only the δ-dodecalactone was labelled from the added radioactive substrate and no evidence of labelling was apparent in the other hydroxy acids present in the samples.

Fig. 2 illustrates the trends in specific radioactivities of the fatty acids esterified in glyceride form in the resulting milk lipids after administration of the labelled substrate. The maximum specific radioactivity occurred in the C_4 acid, and then decreased as chain length of the fatty acids increased. These results demonstrate that the δ-hydroxy acid contributes carbon to the fatty acids in the glycerides of milk fat; however, the trend in specific radioactivity is reversed when compared with the specific radioactivities derived from [^{14}C]acetate (Fig. 1). This incorporation into the C_4, C_6 and C_8 fatty acids is similar to that found after [1-^{14}C]butyrate administration (Bines & Brown, 1968; Ahrens & Luick, 1964). The specific

Table 1. *Description of experiments and substrate incorporation*

Expt. no.	Labelled substrate	Specific radioactivity of substrate	Milking time (hr.)	Incorporation into lipid (%)
1	Sodium [1-^{14}C]acetate	1·0mc/41mg.	5, 10	16
2	Sodium δ-hydroxy[1-^{14}C]laurate	0·05mc/113mg.	3, 6, 9	17
3	Sodium [1-^{14}C]laurate	0·50mc/32mg.	3, 6, 9	21

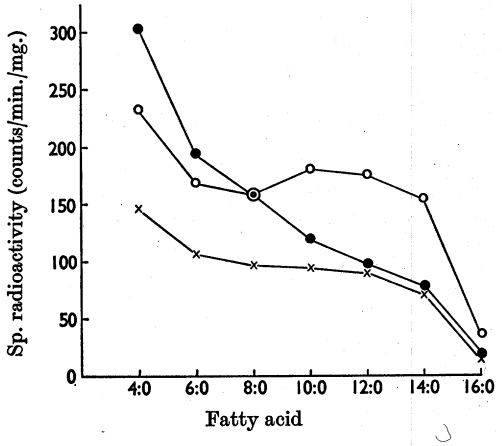


Fig. 2. Expt. 2: specific radioactivity of milk triglyceride fatty acids after intramammary infusion of sodium δ-hydroxy[1-^{14}C]laurate. Samples were taken 3hr. (●), 6hr. (○) and 9hr. (×) after administration of δ-hydroxy-[1-^{14}C]laurate.

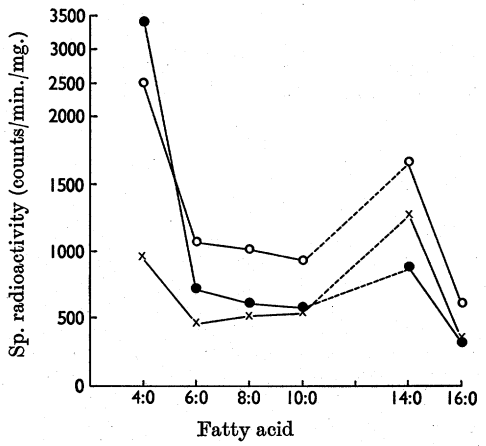


Fig. 3. Expt. 3: specific radioactivity of milk triglyceride fatty acids after intramammary infusion of sodium [1-^{14}C]-laurate. The broken line indicates no results presented for the infused laurate. Samples were taken 3hr. (●), 6hr. (○) and 9hr. (×) after administration of [1-^{14}C]laurate.

radioactivity of the laurate compared with those of the C_4–C_{16} fatty acids demonstrates that the labelled δ-hydroxylaurate is not the exclusive precursor and preferential source of laurate.

Sodium [1-^{14}C]*laurate infusion.* The unique and extensive manner in which δ-hydroxy[1-^{14}C]laurate was utilized in lipid metabolism in the mammary tissue necessitated a further experiment to determine whether the δ-hydroxy fatty acid is an intermediate in the synthesis or the degradation of the corresponding fatty acid. Synthesis of ester lipid classes of milk within the mammary gland from infused saturated fatty acids has been well documented (Patton, McCarthy, Evans, Jensen & Gander, 1962; McCarthy & Patton, 1963). Similarly, in the present study, after sodium [1-^{14}C]laurate infusion, the specific radioactivities of the triglyceride fraction increased from 3hr. to a maximum at 6hr. and decreased thereafter. Of the tracer radioactivity 19% was incorporated into the saturated esterified fatty acids. Understandably most (83%) of this radio-activity was contributed by esterified [1-^{14}C]laurate; however, the remainder (17%) was found in the even-numbered saturated fatty acids (C_4–C_{16}) other than laurate. When the saturated fatty acids, excluding laurate, in these samples were analysed, it was evident that the trend in specific radioactivities was similar to that of the fatty acids after sodium δ-hydroxy[1-^{14}C]-laurate infusion (Fig. 3), i.e. decreasing with increasing chain length of the fatty acid. The increase in specific radioactivity of myristate is attributed to chain elongation (Wakil, 1961) from the highly labelled laurate that was infused. Consecutive trapping of myristate from the gas–liquid-chromatographic column and rechromatographing with subsequent trapping indicated that the high levels of radioactivity in this acid were not due to tracer contamination from the labelled laurate.

Analyses of the hydroxy acid-containing glyceride fraction for each milk sample demonstrated that the γ- and δ-hydroxy acids were significantly labelled (Fig. 4) and characterized by high specific radioactivities (Table 2) as compared with the esterified fatty acids exclusive of C_{12} (Fig. 3). The high specific radioactivities of these compounds in the 3hr. milk-fat sample strongly imply that the saturated fatty acids (in this case [1-^{14}C]laurate) are

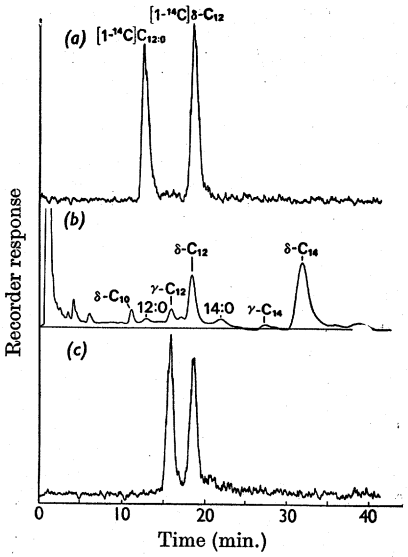


Fig. 4. Gas–liquid radiochromatograms illustrating the presence of ^{14}C in the γ-C_{12} and δ-C_{12} lactones of goat milk fat after intramammary infusion of sodium [1-^{14}C]laurate. (*a*) Authentic compounds; (*b*) mass scan of lactone-rich fraction; (*c*) radiochromatogram of (*b*). Column: 6ft. × 0·25in., U-shaped glass tube packed with 10% (w/w) diethylene glycol adipate+2% (w/w) phosphoric acid on 60–80-mesh Gas Chrom P. Operating conditions: column temp., 198°; detector temp., 240°; flash heater, 225°; carrier gas (helium) flow rate, 120ml./min.; operating voltage for proportional counter, 1800v; quench gas (propane) flow rate, 14ml./min.; combustion tube (CuO) temp., 650°.

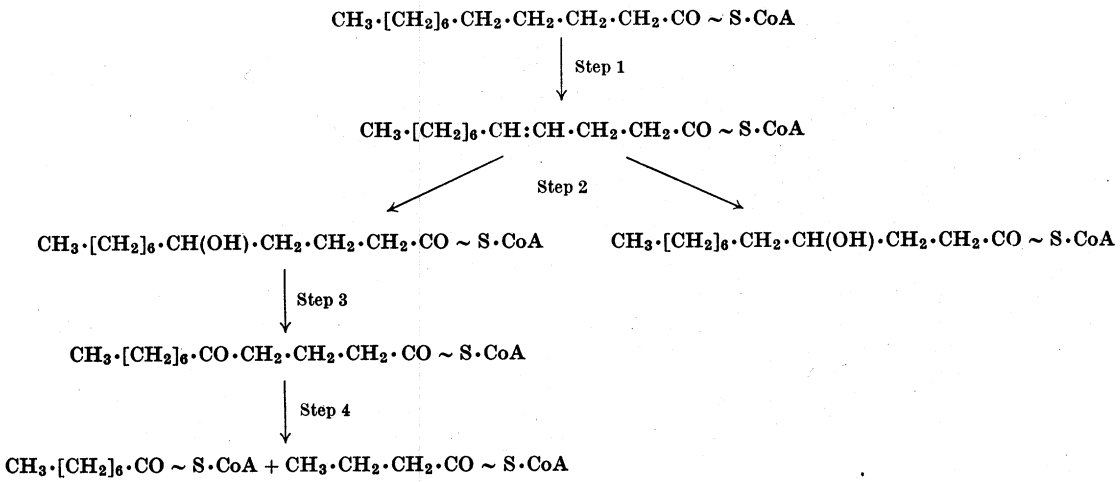
Table 2. *Specific radioactivities of γ- and δ-hydroxylaurate (γ- and δ-lactones) after sodium* [1-^{14}C]*laurate intramammary infusion*

Time after administration (hr.)	Specific radioactivity (counts/min./mg.)	
	γ-Hydroxylaurate	δ-Hydroxylaurate
3	2086260	362104
6	1956300	280420
9	904300	83448

immediate precursors of the γ- and δ-hydroxy fatty acids. Although the reason for the difference in the specific radioactivities of the γ- and δ-lactones is not yet known, it appears that a larger pool size for the δ-lactones with similar rates of metabolism for the two lactones may explain this variation.

DISCUSSION

From the results of this investigation it is evident that endogenous oxidation of the aliphatic saturated fatty acids may occur at the γ- and δ-carbon atoms in such a way that C_4 units are split off. These in turn may contribute to fatty acid biosynthesis in the mammary gland. This mechanism of oxidation is denoted as δ-oxidation as illustrated in Scheme 1. Fatty acids derived from acetate as their thiol esters undergo a dehydrogenation (Scheme 1, step 1) to form a $\gamma\delta$-unsaturated acyl-CoA derivative. The enzymes and cofactors for this entire series of reactions will not be discussed here, but may be similar to those reported necessary for the β-oxidation pathway (Wakil, 1959; Stern, Del Campillo & Raw, 1956; Lynen & Ochoa, 1953). The $\gamma\delta$-unsaturated acyl-CoA esters are subsequently hydrated to form both the γ- and δ-hydroxyacyl-CoA derivatives (Scheme 1, step 2). At this juncture in the reaction, apparently these intermediates may be directed to a very limited extent into milk triglycerides. Alternatively, the δ-hydroxyacyl-CoA compound is then oxidized to δ-oxoacyl-CoA (Scheme 1, step 3). These oxo acids were not isolated during this study; however, trace quantities of the γ- and δ-oxo fatty acids have been reported to occur in milk fat and in proportions consistent with the amounts of the hydroxy acids (Van der Ven, 1964). During the last step of the δ-oxidation pathway the δ-oxoacyl-CoA ester is cleaved (Scheme 1, step 4) to yield an acyl-CoA and butyryl-CoA derivative. It is postulated therefore that this C_4 acyl-CoA derivative is subsequently available to enter a biosynthetic pathway for the formation of primarily the C_4–C_8 fatty acids of milk triglycerides. Correlatively, a C_3 unit also may result from the oxidation and cleavage of the γ-hydroxyacyl-CoA and subsequently contribute to odd-numbered fatty acids. The quantitative significance of this C_4 contribution cannot be estimated from these results since the role of β-hydroxybutyrate supplied via the circulating blood to the gland also must be considered. Possibly the requirement of this C_4 unit in biosynthesis is dependent on the lipid demand in the mammary gland. The widespread occurrence of δ-oxidation in mammalian lipid systems is implied from the presence of the δ-hydroxy fatty acids in milk lipids and adipose tissue of both ruminant and non-ruminant species (Dimick *et al.* 1966*b*). Although carbon-by-carbon degradation analysis of ^{14}C-labelled intermediates and studies of the specific enzymes involved will render understanding of the δ-oxidation scheme more precise, for the moment it is evident that fatty acids oxidized by this route are yielding carbon to a pathway of fatty acid synthesis other than that via acetyl-CoA. These findings appear to confirm the postulation that there are at least two pathways of fatty acid synthesis in the lactating mammary gland (Hele & Popják, 1958; Hele, 1958; Smith & Dils, 1966).



Scheme 1. Proposed pathway for δ-oxidation of saturated fatty acids. Steps 1–4 are discussed in the text.

Moreover, our work reveals the importance of one pathway of fatty acid catabolism in the mammary gland. It now seems particularly appropriate to raise the question whether observed β-oxo acids in milk triglycerides (Boldingh & Taylor, 1962) are intermediate products in the biosynthesis (Lawrence & Hawke, 1966) or β-oxidation of milk fatty acids.

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